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METAL REGULATION AND MOLTING IN THE BLUE CRAB, *CALLINECTES SAPIDUS:* METALLOTHIONEIN FUNCTION IN METAL METABOLISM

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ABSTRACT

We recently demonstrated that zinc, copper, and hemocyanin metabolism in the blue crab varies as a function of the molt cycle. To extend these observations, and better delineate metal metabolism in marine crustaceans, we have conducted experiments to determine if environmental temperature and season of the year affect concentrations of hemocyanin and copper in the hemolymph and copper and zinc in the digestive gland. Overwintering, cold water crabs (6°C) had decreased hemocyanin and copper in the hemolymph and normal zinc and copper in the digestive gland with respect to summer crabs collected at 20–30°C. When these crabs were warmed to 20°C and fed fish for three weeks, they showed increases in the concentrations of copper in the digestive gland, and copper and hemocyanin in the hemolymph. In addition, a change from a zinc to a copper-dominated metallothionein was found in a majority of the warmed crabs, suggesting the involvement of copper metallothionein in the resynthesis of hemocyanin. Based on these observations and previous data (Engel, 1987) a conceptual model of copper and zinc partitioning in the blue crab has been constructed. In this model, metallothionein has an important role in metal regulation both during molting and in the changes related to season of the year. Metallothionein-bound copper and zinc appear to be regulated at the cellular level for the synthesis of metalloproteins, such as hemocyanin (copper) and carbonic anhydrase (zinc), both of which are necessary for normal growth and survival. Finally, we present evidence showing that copper metallothionein can directly transfer its metal to the active site of apohemocyanin. Copper insertion seems to precede the formation of viable oxygen binding sites.

INTRODUCTION

Studies of the reputed function of metallothionein in marine organisms have been concerned primarily with its role in detoxifying elevated concentrations of trace-metals accumulated from polluted environments (Roesijadi, 1981; Engel and Brouwer, 1984; and George and Viarengo, 1985). These reviews discussed the potential constitutive or regulatory function of metallothionein in metal metabolism, but emphasized its role in detoxifying metals.

Previously we alluded to the possibility that metallothioneins may play a role in organismal and cellular metal metabolism in marine species (Engel and Brouwer,

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1984; Engel and Roesijadi, 1987). Such suggestions also have been made concerning zinc and copper metabolism in mammals. Cousins (1982, 1985) discussed the role of metallothionein in zinc metabolism in rats. The observation that glucocorticoid hormones can significantly alter zinc metabolism and increase metallothionein synthesis in the liver without the administration of exogenous zinc also supports the hypothesis that metallothionein is active in normal metal metabolism (Karin, 1985). More recently Petering and Fowler (1986) discussed the normal or constitutive aspects of metallothionein synthesis and turnover in mammals, and correlations also were made with non-mammalian organisms. There is a growing body of evidence, therefore, that metallothioneins are indeed involved in the regulation of metal metabolism.

Recently it was demonstrated that blue crabs collected from unpolluted environments significantly alter tissue metal concentrations and the metal composition of metallothionein during the molt cycle (Engel, 1987). These studies clearly demonstrated that metallothionein in a marine crustacean is actively involved in normal physiological and biochemical processes of metal regulation at the cellular level that control growth and reproduction. Additionally, blue crab metallothionein also is associated with cellular metal detoxification and sequestration (Brouwer *et al.*, 1984; Engel and Brouwer, 1984).

Two series of experiments were performed to explore further the role of metallothionein in metal metabolism. The first series of experiments examined the effect of overwintering on the metal metabolism of the blue crab at both the tissue and cytosolic level. The second series of experiments measured the ability of metallothionein to donate copper for activation of apohemocyanin *in vitro*. In addition, we discuss how metallothionein and the metals bound to it relate to the physiological and biochemical changes that occur during molting. We also propose a model for the direct involvement of metallothionein as a metal donor in the synthesis of hemocyanin and zinc enzymes.

Materials and Methods

All crabs used in these experiments were captured in the vicinity of Beaufort, North Carolina, by commercial fishermen. A group of ten intermolt (C_4) male blue crabs (*Callinectes sapidus*) were obtained in February 1986, and were maintained in the laboratory at ambient temperature and salinity (6°C, 30‰). After a week of acclimation, five crabs were taken for hemolymph and tissue samples. The remaining five crabs were held for an additional three weeks, and water temperature was allowed to increase to about 20°C in 10 days. During the three week period the crabs were fed chopped fish every other day. At the end of three weeks the remaining five crabs were killed and hemolymph and digestive gland samples were taken.

Tissue metal measurements

The concentrations of copper and zinc were determined in samples of digestive gland and hemolymph from individual blue crabs. The hemolymph samples were collected by severing the fifth pereiopod at the meropodite and collecting the fluid in a polyethylene vial. A portion of the hemolymph was taken for metal analysis and the remainder was used for determination of hemocyanin concentration. The crabs were killed by removing the carapace, and the digestive gland was dissected out and used for total metal measurements and cytosolic metal determinations. The tissue

TABLE I

Amino acid composition of blue crab and lobster metallothionein (Residues/6500 Daltons)

	Blue crab			Lobster
	CdMT[a] gill	CdMT[b] digestive gland	ZnMT[b] digestive gland	CuMT[c] digestive gland
Cysteine	18	17	18	18
Asp/Asn	4	4	4	3
Thr	3	5	5	4
Ser	7	5	6	5
Glu/Gln	8	7	6	4
Pro	5	6	5	6
Gly	5	7	6	5
Ala	2	3	3	3
Val	1	1	1	—
Met	—	—	—	—
Ile	—	—	—	—
Leu	—	1	1	—
Tyr	—	—	—	—
Phe	—	—	—	—
His	1	—	—	—
Lys	7	7	7	8
Arg	1	1	1	1
	62	64	63	57

[a] Brouwer *et al.* 1984.
[b] Brouwer unpub. results.
[c] Brouwer *et al.* 1986.

that was used for determination of cytosolic distribution of metals was frozen rapidly and stored in a freezer at −70°C.

Tissue samples used for metal analysis were oven dried at 100°C for 48 h and wet ashed with concentrated HNO_3 at 90°C. Residue was dissolved in 0.25 *N* HCl and concentrations of copper and zinc were measured using flame atomic absorption spectrophotometry. Preparative and measurement techniques were calibrated against the National Bureau of Standards, Oyster Reference Material #1566.

Apohemocyanin reconstitution experiments

We have shown that the digestive gland of the American Lobster, *Homarus americanus*, contains an abundant supply of copper-metallothionein (Engel and Brouwer, 1986). The amino acid composition of the purified metallothionein from lobster is similar to that of the blue crab (Table I). In view of this similarity, and the relative ease with which it can be isolated from the lobster, we have used lobster digestive gland as the source of copper metallothionein in our apohemocyanin reconstitution experiments.

Hemocyanin and copper metallothionein, to be used in copper transfer experiments, were prepared as described previously (Brouwer *et al.*, 1986). Hemocyanin concentration was calculated from the optical density at 280 nm, using $E^{1\%}_{1\,cm} = 14.3$ and a value of 75,000 for the molecular weight of a single oxygen-binding site carrying subunit (Nickerson and Van Holde, 1971). Apohemocyanin was prepared by mixing

hemocyanin in 50 m*M* Tris pH 8, 10 m*M* $CaCl_2$, with an equal volume of buffer containing 20 m*M* KCN. To prepare hemocyanin samples with different amounts of bound copper, the protein was either incubated with KCN for 10 minutes at room temperature, or dialyzed for 30 minutes against 20 m*M* KCN, followed by removal of the KCN on Sephadex G-25. Reconstitution of apohemocyanin was performed by mixing the apoprotein with purified copper metallothioneins in 50 m*M* Tris pH 8, 10 m*M* $CaCl_2$, in the absence of oxygen.

Copper insertion into the active site of the apoprotein was measured by fluorescence spectroscopy. Apohemocyanin was excited at 280 nm and the quenching of the tryptophan fluorescence, which accompanies copper incorporation, was monitored at 340 nm with a SPEX Fluorolog fluorescence spectrophotometer in the ratio mode. The concentration of functionally active oxygen binding sites was determined from the intensity of the copper-oxygen charge transfer band at 340 nm after addition of O_2 to the degassed incubation mixture.

Results

Effect of overwintering on metal partitioning

Differences were observed in the concentrations of copper in hemolymph and digestive gland samples among the three groups of intermolt C_4 crabs that were examined (summer, 1985; winter-cold, 1986; and winter-warmed, 1986). In the hemolymph there was a correlation between the physiological condition of crabs and the concentrations of hemocyanin and copper (Fig. 1). Both summer and warmed hard crabs had hemocyanin and copper concentrations that were higher than the cold crabs, but only the difference between the copper concentrations in summer and cold hard crabs was significant ($P < .05$). Zinc concentrations did not change significantly ($P > .05$) among the three groups of crabs (Fig. 1), and did not appear to be positively correlated with hemocyanin concentration. In the digestive glands there was no significant difference ($P > .05$) in concentrations of copper between the summer and cold water crabs, but there was a significant ($P < .05$) increase in the crabs that were warmed (Fig. 2). Once again zinc concentrations did not show significant changes ($P > .05$). The large increase in copper concentration in the warmed crabs is correlated with the observed increase in hemocyanin in the hemolymph.

The elution profiles obtained after gel-permeation chromatography of the cytosol from digestive glands of cold and warmed crabs showed differences in metals bound to metallothionein. Among the cold water crabs four of five had metallothionein peaks that contained primarily zinc, while three of four (*i.e.*, one chromatographic sample lost) of the warmed crabs had metallothioneins that contained primarily copper (Fig. 3-II and III). Thus, the majority of cold water crabs had Cu/Zn ratios associated with metallothionein that were reminiscent of premolt animals (*i.e.*, high zinc low copper) while the majority of warmed crabs had patterns similar to those of summer intermolt crabs (*i.e.*, high copper low zinc) (Fig. 3-I). These data show that environmental conditions, physiological state, and feeding can affect tissue metal concentrations and the cytosolic distributions of copper and zinc in blue crabs.

Apohemocyanin reconstitution experiments

Removal of copper from the active site of hemocyanin results in an increase of the intrinsic tryptophan fluorescence of the protein (Fig. 4). This observation allowed us to make a distinction between copper insertion and formation of native functional

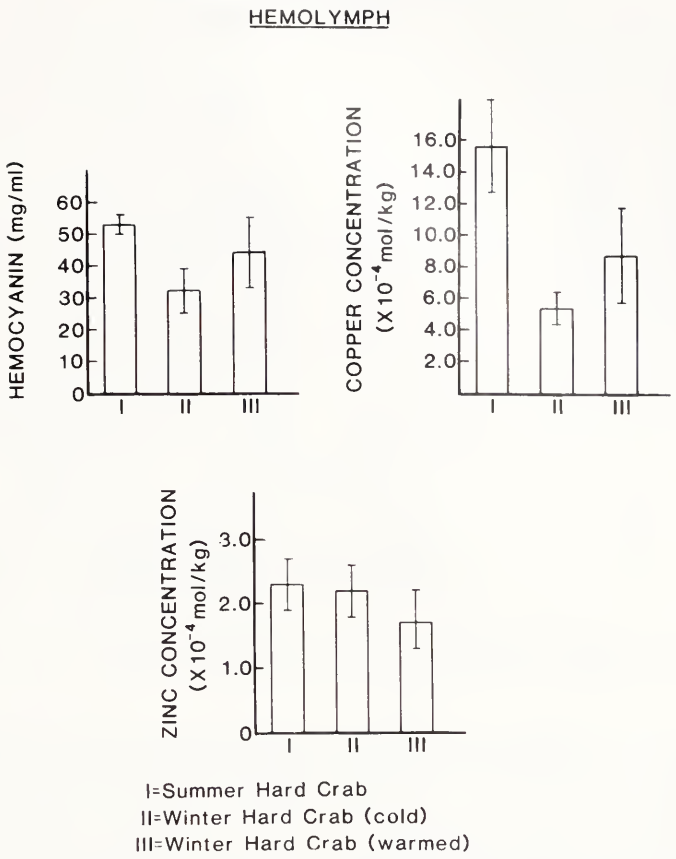

FIGURE 1. Concentrations of hemocyanin, copper, and zinc in the hemolymph of blue crabs collected in the summer (Engel, 1987) and winter. The winter crabs all were collected at the same time. Half (5) were sampled at ambient temperature (6°C) and the other half (5) were warmed to 20°C and fed fish every other day for three weeks. Each histogram represents a mean of five individual crabs plus or minus standard error of the mean.

oxygen binding sites. Both processes can be experimentally followed by fluorescence and absorbance spectroscopy as shown in Figure 5. The data demonstrated that incubation of apohemocyanin with copper metallothionein leads to fluorescence quenching before viable oxygen binding sites are formed, suggesting that copper insertion precedes the formation of biologically active oxygen binding sites (see Discussion).

DISCUSSION

As indicated earlier (Brouwer *et al.*, 1986), one of the proposed functions of copper metallothionein is as a Cu^{+1} donor for hemocyanin synthesis. The present experiment, with dormant and warmed crabs, shows that the predominance of copper on metallothionein among the warmed crabs is associated with the increased levels of hemocyanin in the hemolymph. Our earlier work with blue crabs also suggested a strong correlation between molting, copper metallothionein, and hemocyanin syn-

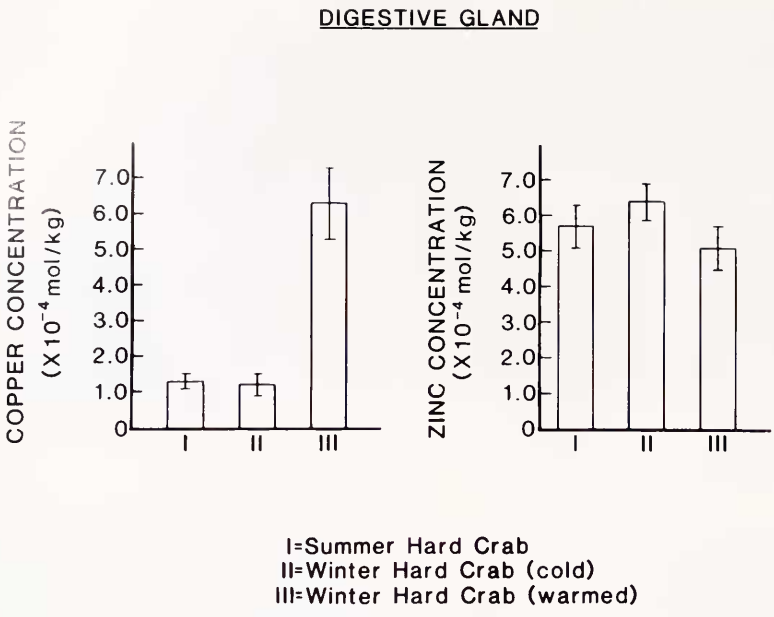

FIGURE 2. Concentrations of copper and zinc in the digestive glands of blue crabs collected in the summer and winter. Further information on handling of the crabs is in Figure 1.

thesis (Engel, 1987). The studies reported in the present paper support that observation and demonstrate that environmentally induced changes and nutrition also can cause changes in the copper/zinc ratios associated with metallothionein. This observation is important because it further emphasizes the possible constitutive role of metallothioneins in normal metabolism.

In the following section we will develop a model of the regulation of copper and zinc partitioning in the blue crab, based on studies by us (Engel, 1987; also present paper), Soumoff and Skinner (1983), and Henry and Kormanik (1985).

The diagrams in Figure 6 display the physiological and biochemical processes involved in crustacean molting and the cyclic and chronological nature of these events. The first of these diagrams (Fig. 6A) depicts the relative duration of the different portions of the molt cycle. The actual timing of events is dependent upon both environmental temperature and the size of the crab (Johnson, 1980). This type of presentation emphasizes the fact that the most dramatic/traumatic changes in the crabs occur over a relatively short period of time. The changes in concentrations of copper and zinc associated with metallothionein are dramatic and provide further evidence as to the dynamic nature of the molting process (Fig. 6B). If it is assumed that metallothionein-bound copper and zinc are associated with metalloprotein and metalloenzyme synthesis, we can predict when hemocyanin and zinc enzyme synthesis occurs during the cycle (Fig. 6C, D). These predictions are tentative and will need to be confirmed in future studies, since there are no direct data available in the literature on these aspects of crustacean physiology.

Copper metallothionein levels during the molt cycle are related directly to hemocyanin concentrations in hemolymph, and inversely to ecdysteroid concentrations in the hemolymph (Fig. 7). The decrease in digestive gland copper metallothionein is

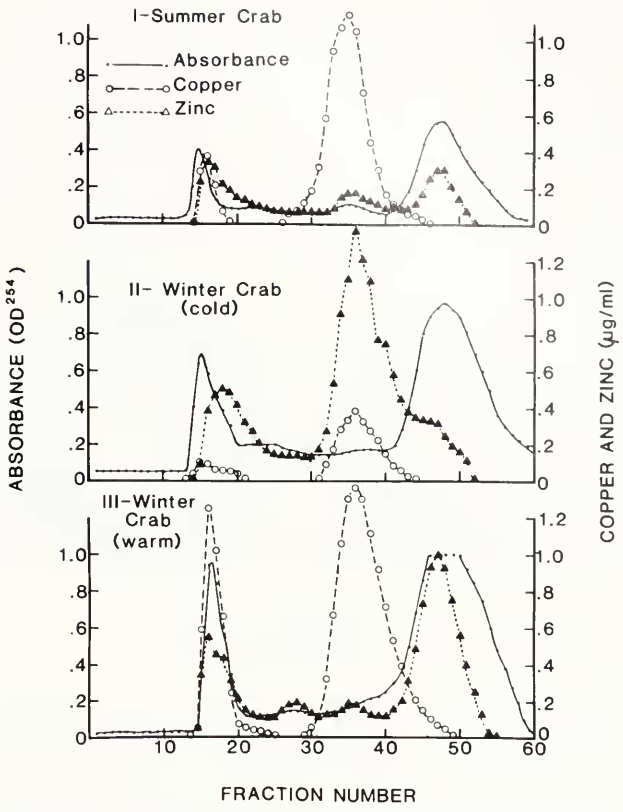

FIGURE 3. Sephadex G-75 elution profiles of digestive gland cytosol prepared from blue crabs collected during the summer and winter. For further information on the crabs see Figure 1. Protein separations were made using 60 m*M* Tris buffer, pH 7.9 with 2 m*M* β-mercaptoethanol with a 2.6 × 60 cm column at a flow rate of 30 ml/h in all three groups of crabs (I, II, III).

correlated with an increase in ecdysteroid titer in blue crab hemolymph (Soumoff and Skinner, 1983). The ecdysteroid concentrations followed the same general pattern for males and immature females throughout the molt cycle. After molt the ecdysteroid level decreases rapidly with concomitant decreases in hemocyanin concentrations. Coincident with these decreases is an increase in copper metallothionein during the A_2 and B_1 stages, followed by an increase in hemocyanin during B_1. Such interrelationships suggest a possible association between the molting hormone ecdysteroid, and the regulation of hemocyanin synthesis and levels of cytosolic copper. It is relevant to emphasize that synthesis of constitutive metallothioneins in mammals is under the control of steroid hormones (Karin *et al.*, 1980 a, b; Karin *et al.*, 1981). No such information exists for the invertebrate metallothioneins. The effect of the molting hormone 20-hydroxyecdysone on metallothionein synthesis in the blue crab is presently under investigation.

Comparisons of zinc metallothionein (Engel, 1987) and ecdysteroid concentrations (Soumoff and Skinner, 1983) and carbonic anhydrase activity (Henry and Kormanik, 1985) during the molt cycle suggest an inter-relationship between these three

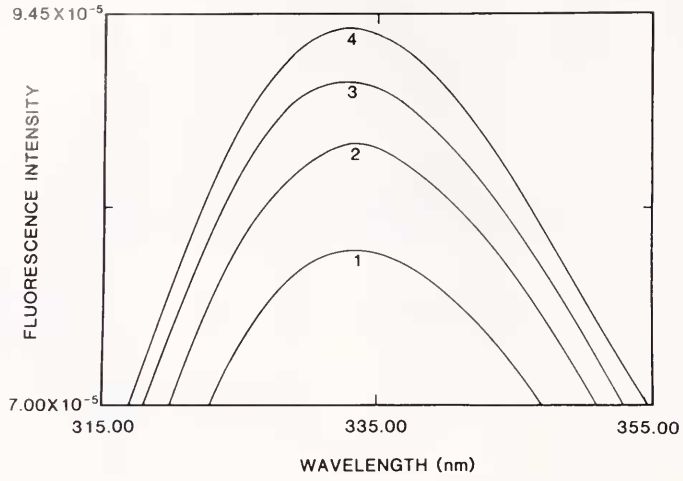

FIGURE 4. Fluorescence intensity of deoxygenated lobster hemocyanin in 50 m*M* Tris pH 8.0 + 10 m*M* $CaCl_2$ as a function of the percentage copper remaining in the active site after dialysis against 20 m*M* Cyanide for 0, 5, 10, and 30 min. (1) 100%, (2) 60%, (3) 30%, (4) 8%. Excitation is at 280 nm.

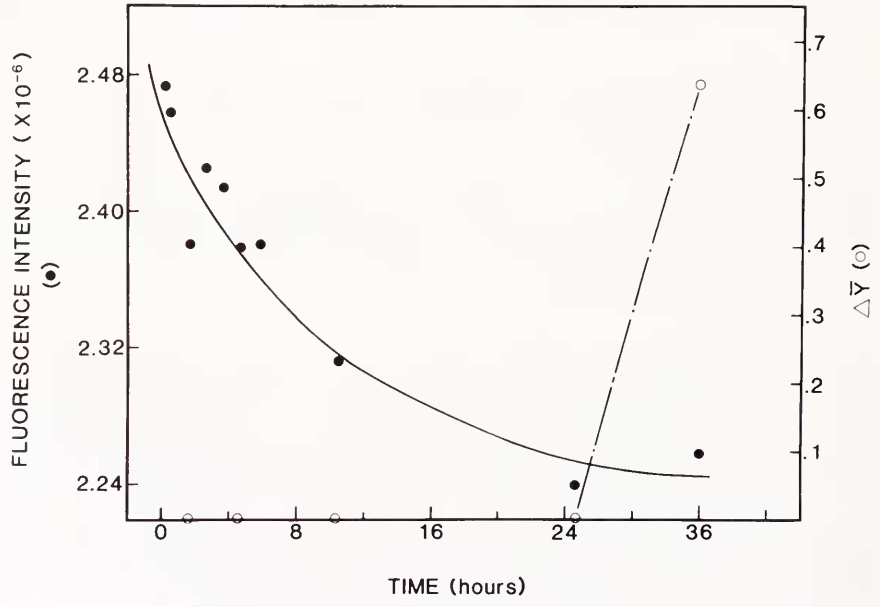

FIGURE 5. Change in fluorescence intensity and oxygen binding capacity (Δ Y) of partial apohemocyanin (5 μM) in 50 m*M* Tris pH 8.0 + 10 m*M* $CaCl_2$ still containing 35% of its original copper, as a function of incubation time with copper-metallothionein (10 μM Cu) in the absence of oxygen. The fluorescence change (copper insertion) precedes the formation of viable oxygen binding sites.

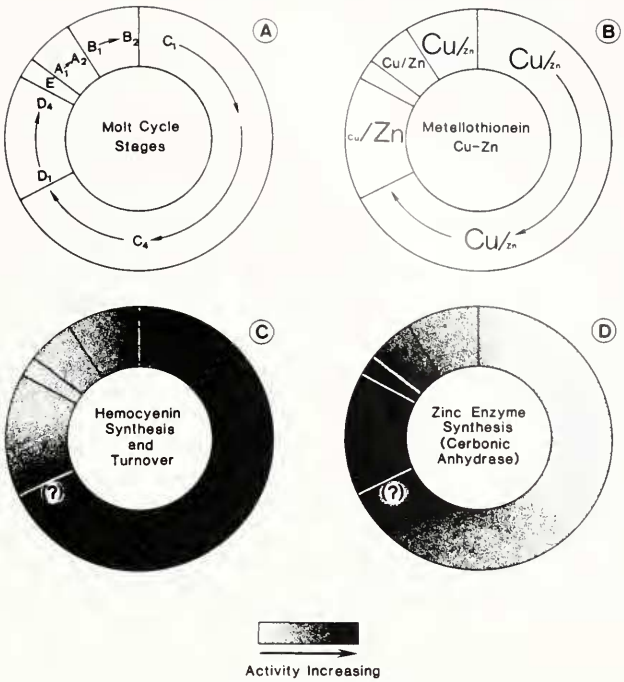

FIGURE 6. Diagrammatic representation of the physiological and biochemical events occurring during the molt cycle of the blue crab. (A) the molt cycle of the blue crab with the duration of each portion indicating time. The designations of the molt stages are: $C_1 \rightarrow C_4$, hard crab; $D_1 \rightarrow D_4$; premolt; E, ecdysis; A_1–A_2, soft crab; B_1–B_2, papershell crab (Mangum, 1985). (B) The relative concentrations of copper and zinc on metallothionein are represented by the size of the copper, Cu and zinc, Zn symbols. (C) and (D) These two figures represent predicted hemocyanin and zinc enzyme synthesis activities generated from previously collected data (Engel, 1987). The degrees of shading are indications of the proposed activities of the biochemical pathways involved in hemocyanin synthesis and turnover, and zinc enzyme synthesis (carbonic anhydrase).

components (Fig. 8). During the premolt period (D_1–D_3) when both zinc metallothionein and ecdysteroid are at their peaks, the new epidermis is being synthesized beneath the existing exoskeleton. At molt both zinc metallothionein and ecdysteroid decrease, and between stages A_1 and A_2 there is an abrupt increase in carbonic anhydrase activity in the newly formed exoskeleton epidermis (Henry and Kormanik, 1985). This rapid increase, which occurs over a period of hours, suggests that the enzyme may be synthesized and present in the new epidermis as an apo-protein, and is not activated by zinc until after molt. Even though the decrease in zinc-metallothionein occurs in the digestive gland and the increase of carbonic anhydrase in the epidermis, these two events may be linked. Possibly some of the zinc bound to metallothionein at the time of molt could be mobilized via the hemolymph to activate the apo-carbonic anhydrase. This hypothesis is attractive since preliminary results from our laboratory have shown the release of zinc from zinc metallothionein during stages A_1 and A_2 (D. W. Engel, unpub. data).

The proposed cycles for copper and zinc-metallothionein (Figs. 7, 8) are speculative, but they are based upon the best available information on the physiological and

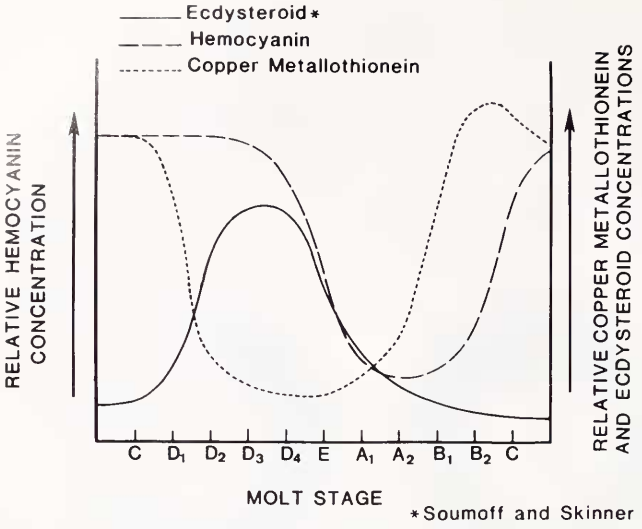

FIGURE 7. A diagrammatic representation of the processes involved in hemocyanin synthesis and turnover, and the interactions between ecdysteroids and copper metallothionein. The data on hemocyanin and copper metallothionein concentrations are from Engel (1987) and for ecdysteroid from Soumoff and Skinner (1983).

biochemical events controlling metal partitioning during molt. These changes are reproducible, and our experiments concerning the effects of thermal changes on metal distributions give further support to the hypothesis that metallothionein is a constitutive metal-binding protein in blue crabs.

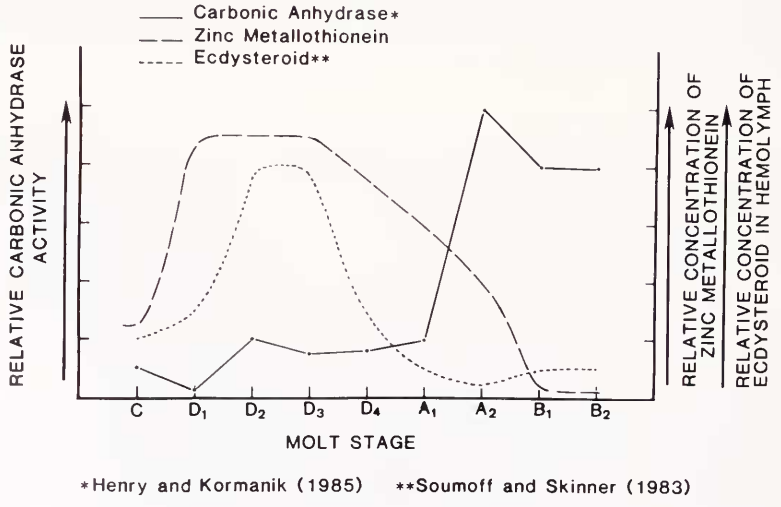

FIGURE 8. A diagrammatic representation of the processes involved in the synthesis of zinc-dependent enzymes and in particular carbonic anhydrase, and how zinc metallothionein and ecdysteroid interact to affect enzyme activity. The data on zinc metallothionein are from Engel (1987), on ecdysteroid from Soumoff and Skinner (1983), and carbonic anhydrase from Henry and Kormanik (1985).

The events and processes described here and in an earlier publication (Engel, 1987) do not address the question of control of the molt cycle and metal turnover. During the molt cycle there are pronounced changes in the cytosolic distribution and tissue concentrations of metals and accompanying changes in the hemolymph ecdysteroid concentrations. Studies by Singer and Lee (1977) suggest that the hemolymph hormonal levels are modulated by changing MFO (mixed function oxygenases) activity in the antennal gland. These authors demonstrated that MFO activity in the gland also varies with stages of the molt cycle. This activity is negatively correlated with the ecdysteroid levels (Soumoff and Skinner, 1983), suggesting that the MFO system controls steroid/hormonal concentrations in molting blue crabs, which in turn may affect metal partitioning as described in this paper.

Further evidence for metallothionein's metal regulatory function comes from the *in vitro* hemocyanin reconstitution experiments. Apohemocyanin can only be reconstituted with Cu^{+1} (Konings *et al.*, 1969; Lontie and Witters, 1973). Since copper binds to metallothionein as Cu^{+1}, and since copper metallothionein levels and hemocyanin biosynthesis seem to be linked *in vivo*, we initiated a study of hemocyanin-copper metallothionein interaction *in vitro*. The data presented in Figure 4 show that the intrinsic tryptophan fluorescence of lobster hemocyanin strongly depends on the amount of Cu^{+1} bound to the active site. This is in line with the observations that several crustacean hemocyanins contain tryptophan residues in close proximity to the binuclear copper site (Gaykema *et al.*, 1984; Linzen *et al.*, 1985). This property allowed us to make a distinction betwen Cu^{+1} incorporation into the active site of apohemocyanin and the formation of native functional oxygen binding sites. It is evident from Figure 5 that the quenching of tryptophan fluorescence, observed when apohemocyanin is incubated with copper metallothionein, precedes the formation of biologically active oxygen binding sites. This strongly suggests that the copper transfer process is followed by a slow reordering of the tertiary structure of the copper sites to the native configuration. Similar sequences have been demonstrated for the reconstitution process of many Cu^{+2} proteins where binding of copper to the active site is followed by a slow return of the protein to its biologically active state (Kertesz *et al.*, 1972; Morpurgo *et al.*, 1972; Rigo *et al.*, 1978; Marks *et al.*, 1979; Blaszak *et al.*, 1983). This observation may also explain why reconstitution of apohemocyanin with copper metallothionein can only be accomplished under anaerobic conditions (Brouwer *et al.*, 1986). The Cu^{+1} in the distorted sites is not capable of combining reversibly with oxygen. Interaction of oxygen with Cu^{+1} under these conditions results in oxidation of metal. These Cu^{+2}-sites will not bind oxygen and are lost for detection by absorbance spectroscopy. This hypothesis is presently under further investigation.

The studies described in this paper have demonstrated that marine crustacea are excellent model systems to study the role of metallothionein in copper/zinc metabolism on an organismal, cellular, and molecular level. Only when this function of metallothionein is fully understood will it be possible to assess its value as a metal-detoxifying protein.

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